Distribution of the members of *Anopheles gambiae* and pyrethroid knock-down resistance gene (kdr) in Guinea-Bissau, West Africa.

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Résumé : **Distribution des espèces du complexe *Anopheles gambiae* et du gène de résistance aux pyréthrinoides (mutation kdr) en Guinée-Bissau, Afrique de l'Ouest.**

Une étude entomologique a été réalisée en 2002 dans quatre localités couvrant différents faciès écologiques de la Guinée Bissau : Buba Tombao (forêt), Gabu (savane), Cacheu (mangrove) et Bissau (urbain) afin i) d'étudier la distribution des membres du complexe Anopheles gambiae (Diptera: Culicidae) *ii) d'évaluer le statut de résistance de ces vecteurs du paludisme aux insecticides (perméthrine 0.75%, DDT 4%) et enfin iii) de rechercher la présence et la distribution de la mutation kdr au sein de ces populations.*

Les femelles de moustiques adultes issues de captures matinales à l'intérieur des maisons ont été testées suivant les procédures OMS (kit de bio essai et papier imprégné) afin d'évaluer leur statut de résistance aux insecticides. Les spécimens testés ont été identifiés et caractérisés pour la présence de la mutation kdr par PCR.

En Guinée Bissau, dans les localités étudiées, le complexe An. gambiae *est dominé par* An. gambiae *s.s. (avec les deux formes moléculaires S et M représentées) vivant en sympatrie sur le littoral avec une faible proportion d'*An. melas*. Les populations d'*An. gambiae *s.s. exposées aux deux insecticides se sont révélées sensibles quelle que soit leur provenance. La mutation kdr Leu-Phe a été détectée en de très faibles fréquences seulement dans deux localités situées respectivement en zone urbaine (Bissau) et en savane (Gabu). Cette mutation est présente uniquement dans la forme moléculaire S à Gabu (avec une fréquence allélique de 0.14) et dans les deux formes moléculaires M et S à Bissau avec des fréquences alléliques respectives de 0.06 et de 0.02.*

*Ces résultats suggèrent que les populations d'*An. gambiae *s.s., vecteur le plus fréquent du paludisme en Guinée Bissau, demeurent encore sensibles aux pyréthrinoides et au DDT 4%. Ce statut de sensibilité ainsi que la fréquence des gènes de résistance tel que le kdr doivent être surveillés dans le futur particulièrement dans les zones urbaine et de savane soumises à une utilisation intensive d'insecticides.*

Anopheles gambiae *s.s.*
gène kdr
sensibilité aux insecticides
Buba Tombao
Gabu
Cacheu
Bissau
Guinée Bissau
Afrique de l'Ouest

Summary:

An entomological survey conducted in 2002 in Guinea Bissau aimed i) to study the distribution of the members of Anopheles gambiae Giles complex (Diptera: Culicidae) *throughout four ecological areas extended from mangrove to savannah ii) to evaluate the insecticide susceptibility status of these malaria vectors exposed to permethrin 0.75% and DDT4%, and finally iii) to investigate the occurrence and the spread of the Leu-Phe knock down resistance (kdr) gene associated with pyrethroid and DDT resistance within these vector populations.*

Adult female mosquitoes issued from indoor morning collections were tested using WHO procedures, test kits and impregnated papers to assess their insecticide susceptibility status. Tested specimens were identified by PCR assays and characterized for the kdr gene.

Malaria vectors were mainly dominated elsewhere by An. gambiae *s.s. (both S and M molecular forms) living in sympatry with low proportion of* An. melas *in the littoral.* An. gambiae *s.s. tested populations were fully susceptible both to permethrin 0.75% and to DDT 4% irrespective to their location and ecotypes. The Leu-Phe kdr mutation was detected at low frequency only in two sites respectively urban (Bissau) and Guinea-savannah (Gabu) areas. It occurred only in the S molecular form in Gabu (at the frequency of 0.14) and both in the S and M molecular forms in Bissau at the frequency of 0.06 and 0.02 respectively. These results suggested that the populations of* An. gambiae *s.s., the most frequent malaria vector in Guinea Bissau, still remain cross-susceptible to pyrethroids and DDT. This susceptibility status and the frequency of resistance mechanism such as the kdr mutation must be monitored in the future particularly in the urban and savannah areas with continuous and intensive use of insecticides.*

Anopheles gambiae *s.s.*
kdr gene
insecticide susceptibility
Buba Tombao
Gabu
Cacheu
Bissau
Guinea Bissau
West Africa

Introduction

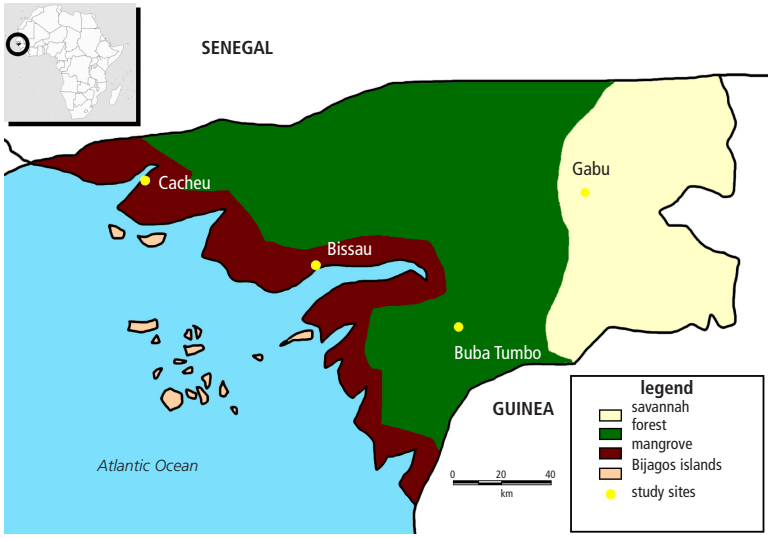
Malaria is one of the most serious vector-borne diseases affecting millions of people, mainly in Africa. More than 90% of the deaths, resulting from malaria occurred particularly in children aged 1-5 (30). Despite the huge burden and the absence of a viable vaccine for the moment , few tools are available to control this disease. Current policy options include direct treatment of patient with anti-malarial drugs together with preventive method aiming at reducing human vector-contact by indoor sprays as well as using mainly insecticide-treated nets (ITNs). However big efforts have been made in many African malaria-endemic countries such as "Roll Back Malaria" (RBM) partnership, but only less than 2% of children sleep under ITNs (31). Indeed the ITNs were considered as one of the major tools in the global control of malaria focused on the intervention targeted at adult anophelines. Its efficacy was proved through many studies mentioning the reduction of mortality in the target populations such as in 1-5 year-old children (5, 11). Unfortunately knock-down resistance (kdr) conferring resistance to pyrethroids and cross resistance to DDT as first reported in *Anopheles gambiae* s.s. populations in Côte d'Ivoire (12), has been observed, spreading out mainly in West Africa from Mali to Nigeria (2, 3, 4, 8, 29, 33). This mutation resulting from one single point mutation (Leucine TTA to Phenylalanine TCA) was probably associated prior with the intensive use of DDT and lately with pyrethroids, used for crop protection particularly in cotton areas and in lower proportion to the domestic use of insecticides against nuisance (4, 8). The Leu-Phe kdr mutation is detected at high frequency in the M molecular form of the *An. gambiae* s.s. populations present on the littoral of West Africa especially in Benin and Côte d'Ivoire (Chandre, com. pers.). Inland, in the savannah zone, the kdr mutation predominates in the S molecular form of *An. gambiae* being rare either in the M molecular form or in *An. arabiensis* populations (9).

Although some studies indicated that ITNs remained efficient to carry out vector control in kdr-pyrethroid resistance area (6, 18) some decrease of ITNs efficiency has been observed recently among kdr-M molecular form of *An. gambiae* s.s. in coastal Benin (23).

If some countries in West Africa, due of the implementation of scientific and technical logistics, are able to provide information on malaria transmission, distribution of members of the *An. gambiae* complex and vector resistance status including distribution of the kdr gene, little is known in other countries such as Guinea Bissau, Liberia or Sierra Leona facing social and political trouble. In the context of generalized spread of pyrethroid-treated nets (ITNs) and general use of pyrethroids in cotton crops, it is crucial to better know the specific identity and resistance status of vector populations in each country and ecological zones. This study initiated by the African Network in Vector Resistance (ANVR) supported by WHO, was dedicated to the investigation of the occurrence and spread of Leu-Phe kdr resistance in poor investigated countries of West Africa such as Guinea Bissau where little is done in malaria vector characterization and control. We report here the distribution of the members of *An. gambiae* complex and the spread of the Leu-Phe kdr gene within these vector populations across different ecological zones in Guinea Bissau.

Figure 1.

Study sites location in Guinea Bissau (West Africa).
Sites d'étude en Guinée Bissau (Afrique de l'Ouest).



Materials and methods

Location of sampling sites

Guinea Bissau is a small country located in Western Africa, bordering the North Atlantic Ocean, between Guinea and Senegal (12°N, 15°W). Its total area covers 36,120 km² with 1,470,000 inhabitants. This country is swampy along its western coast and low-lying further inland. Four sites corresponding to different ecological set-ups including urban area, mangrove, forest and Guinea-savannah were sampled from September to October 2002 (figure 1).

Bissau capital of Guinea Bissau (15°28'05"W, 11°54'32"N) is an urban agglomeration located in the littoral and surrounded by mangroves and flat lowlands affected by the daily tides. Rice growth is the main agricultural activity and uses few insecticides for crop protection.

Cacheu (16°13'01"W, 12°14'56"N) is also located in the littoral mainly dominated by the mangrove. Here rice is also the main agricultural speculation. Buba (14°50'35"W, 11°39'50"N) is located in the forest with manioc and maize as the predominant crops.

Gabu (14°18'07"W', 12°11'56"N) located in the eastern Guinea-savannah region of the country is dominated by groundnuts and millet growth with some extended fruit trees garden. Cotton is also grown in this area involving the use of pyrethroids replacing DDT formerly used for crop protection.

The country is located in the tropical Guinean climatic zone, generally hot and humid. There are two distinct seasons: monsoonal-type rainy season (June to November) with south-westerly winds; dry season (December to May) with north-easterly harmattan winds. The average annual rainfall ranges from 2,000 mm in the south forest coast to 1,000 mm in the north-east savannah. Malaria is wide spread and holoendemic in the country with a peak of transmission at the end of the rainy season (October-November). A preliminary study has shown that the *Plasmodium* index in children 2-9 years old in villages of the north-western littoral zone in Guinea-Bissau ranged at the end of the rainy season between 44% and 79%. The malaria vectors in the studied villages were identified as *An. gambiae* s.s. and *An. melas* (19). Both species belonging to the *An. gambiae* complex are anthrophaphic,

endophagic and endophilic, and transmit efficiently human malaria parasites (19, 25).

Mosquito collection

Mosquitoes were collected from 4 sites at the end of the rainy season (September and October 2002). Because of the difficulties met during the study period due mainly to the rainfall diluting the larva populations and the swampy environment of most areas (about 20% of inlands were flooded during the rainy season), indoor resting adult females were collected very early in the morning (5h-7h) from human dwellings using manual aspirators and torches. They were kept alive and brought to the National Laboratory of Public Health in Bissau where the insecticide tests were carried out. We noted that un-impregnated bednets of varying quality, but usually of poor condition, were used in all houses across all the ecological zones by most of the people. This use is directly linked to the highest mosquito nuisance felt by the inhabitants more particularly at this time of the year. It could facilitate the use of ITNs against malaria transmission at a large scale in the country.

Insecticide susceptibility test

The insecticide susceptibility tests were performed on wild age-undetermined females composed of gravid and half gravid anophelines collected in bedrooms as described above using 0.75% permethrin (cis:trans 25:75) impregnated filter papers as recommended by WHO (32). Only mosquitoes from Bissau were tested to DDT 4% to assess pyrethroid/DDT cross resistance because the number of mosquitoes from other localities was too weak and did not permit to do a bioassay. After the 1-h exposure, the mosquitoes were maintained on 10% sucrose solution and final mortality recorded after 24h. Specimens were preserved individually on desiccated silica gel and identified post-mortem as members of *An. gambiae* s.l. using morphological keys (16, 17).

In addition to mortality recovery period, insecticide knockdown effects were recorded after 10, 20, 30, 40 and 60-minute exposures. 56% knockdown times (KDT_{50} and KDT_{95}) were estimated. They were assigned mortality status, defined as resistant if they showed less than 90% mortality with DDT 4% and less than 95% mortality with permethrin 0.75%. Samples were then kept individually on desiccated tubes after morphological identification (16) for PCR analysis.

To control the quality of the test performed directly with wild age-undetermined mosquitoes in field conditions, the same impregnated papers were tested in the laboratory of Centre Muraz (Burkina Faso) using both standard age (2-3 day old non blood-fed females) and age-undermined females of the "Kisumu" reference strain of *An. gambiae* s.s. This strain has been maintained in the insectarium at the Centre Muraz since 1999 and is 100% susceptible to the diagnostic concentrations of the insecticide used.

PCR analysis

Females exposed to permethrin 0.75% and also to DDT 4% (only in Bissau) and conserved on silicagel tubes were pooled per mortality status. An average of 30 mosquitoes including 10 from the alive group (if any) out of 20 dead mosquitoes were randomly sub-sampled among the samples described above and tested by PCR to i) identify the species within the *An. gambiae* complex (23), ii) characterise the molecular forms (M or S) within *An. gambiae* s.s. (14) and finally iii) to detect the Leu-Phe kdr mutation (20).

Table I.

Insecticide susceptibility test on An. gambiae s.l. females originated from the four ecological zones in Guinea-Bissau and from Kisumu reference strain including standard age and age-undetermined females (WHO tubes).

Test de sensibilité aux insecticides des populations sauvages d'Anopheles gambiae s.l. issues des quatre zones écologiques de la Guinée Bissau et de la souche de référence Kisumu sur âge standard et âge indéterminé (tubes OMS).

study sites	Permethrin 0,75%				DDT 4%			
	n	KDT_{50} (min)	KDT_{95} (min)	M% status	n	KDT_{50} (min)	KDT_{95} (min)	M% status
Bissau Urban area	102	10	20	97% S	93	55	Nd	95% S
Cacheu littoral	121	11	18	100% S	-	-	-	-
Gabu savannah	101	12	22	96% S	-	-	-	-
Buba Tumbo forest	106	12	22	98% S	-	-	-	-
Kisumu «standard age»	105	17	29	98% S	97	25	41	100% S
Kisumu «age-undetermined»	82	14	27	97% S	76	23	44	99% S

n: number of mosquitoes tested (*An. gambiae* s.l.); $KDT_{50}/_{95}$: exposure time in minute during that 50% or 95% of individuals exposed to insecticide were knocked down; M%: percentage of mortality recorded 24h after 1h exposure status: s = susceptible (90%<M≤100%)

Results

Insecticide susceptibility test

The mortality in control group non-exposed (both Kisumu and wild mosquitoes) to insecticide was consistently less than 5% therefore no Abbott correction was necessary during the analysis. The results of the insecticide susceptibility tests showed that 430 mosquitoes collected across the four localities were susceptible to the diagnostic dosage of permethrin 0.75% with a mortality rate ranging from 97% to 100% (table I). The KDT_{50} and KDT_{95} values did not differ significantly from those of the laboratory *An. gambiae* susceptible "Kisumu" strain ($p>0.05$). The mosquitoes from Bissau tested to DDT 4% were also susceptible with a mortality rate reaching 95%. However, the KDT_{50} value was higher than that of the Kisumu strain ($p<0.05$). The test carried out in the Centre Muraz laboratory with the same impregnated papers used for the field evaluation in Guinea Bissau showed that both laboratory 2 to 5 day-old unfed females and age-undetermined females from Kisumu reference strain were also fully susceptible without any difference between the mortality rates ($p>0.05$).

Distribution of *An. gambiae* complex species and molecular forms

Over all 120 mosquitoes fully composed of females exposed to permethrin 0.75% were tested by PCR. The collection was a mix of the molecular M and S forms except in Cacheu (mangrove zone) where *An. melas* were also found at the frequency of 23%. No specimens of *An. arabiensis* were identified in these samples. Indeed in Bissau the urban area and Buba the forest one, the M molecular form predominated reaching respectively 70% and 90%. In contrast both in Cacheu and Gabu respectively in mangrove and Guinea-savannah areas the S molecular form predominated reaching in proportion 54% and 73%. No M/S heterozygote was found.

Table II.

Species and molecular forms identification and frequency of knockdown resistance (Leu-Phe kdr) mutation among specimens of *An. gambiae* s.l. tested in the four ecological zones in Guinea-Bissau.
Identification des espèces et des formes moléculaires du complexe Anopheles gambiae *et distribution du gène kdr dans les quatre zones écologiques de la Guinée Bissau.*

study sites	*An. gambiae* s.l.		*An. gambiae* S form		*An. gambiae* M form		*An. melas*	
	n	Fkdr	%	Fkdr	%	Fkdr	%	Fkdr
Bissau urban area	30	0,03	30	0,06	70	0,02	-	-
Cacheu mangrove	30	0	54	0	23	0	23	0
Gabu Guinea-savannah	30	0,10	73	0,14	27	0	-	-
Buba Tumbo forest	30	0	10	0	90	0	-	-
total	**120**	**0,03**	**41,7**	**0,07**	**52,5**	**0,008**	**5,8**	

n: number of females tested in PCR; Fkdr: allelic frequency of kdr mutation based on the formula (2RR+1RS)/2(RR+RS+SS); %: percentage of species and molecular forms of *An. gambiae* complex tested in PCR

Distribution of kdr gene

On the whole the Leu-Phe kdr gene was found at low frequency averaging 0.07 in the S form and 0.008 in the M form and occurred only in two localities, Bissau (urban) and Gabu (Guinea-savannah). No kdr was detected in *An. melas* specimen. The relatively highest frequency (0.14) of kdr was exclusively observed in the S molecular form at Gabu where also the relative low mortality rate has been obtained with permethrin 0.75%. The two other specimens exhibiting the kdr genotype were found in the urban area from Bissau at the frequency of 0.03. No kdr gene was found in the mangrove and forest sites (table II).

Discussion

Our study confirmed that *An. gambiae* s.s. was the most abundant malaria vector in Guinea Bissau and revealed that the two molecular forms (M and S) lived in sympatry in varying frequencies. Indeed the S molecular form was found largely distributed in the Guinea-savannah and the littoral mangrove but in low proportion in the forest and the littoral-urbanised areas dominated by the M form. *An. melas* was found only in one site (mangrove) because it breeds preferentially in salt water. In West and Central African littoral, both *An. melas* and *An. gambiae* s.s. constituted efficient vectors of *P. falciparum* (2). In extreme environment (mangrove swamps), *An. melas* could be the exclusive present malaria vector (10, 22). Its frequency decreases classically either in urban environment even in littoral as in Bissau city, particularly during the rainy season (15), or inland in forest and savannah zones. Previous studies conducted in the urban area of Bissau have already revealed the predominance of *An. gambiae* s.s. and the rarity of *An. melas* indoors (24, 25). Overall these two species were known to transmit malaria in Guinea Bissau (18).

No *An. arabiensis* was found in our samples due mainly to the sampling period occurring during the rainy season. Indeed in the past, Petrarca et al. (26) collected few individuals of this species only in dry season. In Sudanese savannahs of West Africa *An. arabiensis* is well spread living in sympatry with *An. gambiae* s.s. (7, 20) being the most abundant in Sahelian zone. This malaria vector adapted to dried environment is relatively rare in Guinea-savannah zone. Its absence in Gabu in the present study can be due also to the low sample size and to the sampling methods ie indoor morning collections as *An. arabiensis* is less endophagic and endophilic than *An. gambiae* s.s.. These preliminary results on the malaria vector distribution in Guinea-Bissau agree with reports from other comparable parts of West Africa. It reflects the situation at the end of the rainy season when mosquito populations and malaria transmission usually peak in tropical regions. Although the proportion of mosquitoes collected during the dry season should be lower, the frequencies of *An. gambiae* M form, *An. gambiae* S form and *An. melas* in each of the ecological zone should be comparable (3).

Results of insecticide susceptibility test indicate that the malaria vector populations in Guinea Bissau still remain fully susceptible to pyrethroids and also to DDT although the KDT_{50} and KDT_{95} values obtained with the latter in Bissau suggest a decrease of DDT susceptibility in *An. gambiae* s.s. populations. Even thought the test was done directly on wild age-undetermined females these results agreed with the findings from a relatively recent study in this country reporting the efficacy of ITNs on malaria transmission reported by Jaenson et al. (19).

The molecular detection of the Leu-Phe kdr mutation indicated that the kdr gene is present for the moment at low frequency maintaining the susceptibility status revealed by the bioassay. Indeed compared to the kdr frequencies reported from other West African countries ranging from 80 to 95% such as in Côte d'Ivoire, Benin and Burkina Faso where *An. gambiae* s.s. populations have shown resistance to pyrethroids (1, 4, 8, 13), the frequency of this gene in Guinea Bissau is very low. The finding of kdr gene in the M molecular form was in accordance with reports from other countries in West Africa mentioning the occurrence of this gene in the M molecular form both in littoral and inlands due to genetic introgression from the S molecular form (2, 9). But the presence of the kdr mutation both in urban and Guinea-savannah zones in Guinea-Bissau suggests the possibility of its large-scale spreading in the coming years potentially in the cotton growing savannah areas where the use of pyrethroids is relatively intensive. Although it was a very transversal survey, this study provides basic information enable to contribute to vector control management. That is crucial in a global context where the debates are launching in the large vulgarisation of ITNs and the re-introduction of DDT for indoor spraying (30) to achieve malaria vector control. More extensive studies of vectors are required to support the malaria control programme in Guinea-Bissau including accurate species identification, their behaviour and their role played in malaria transmission.

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